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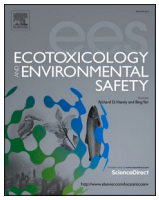

Sodium fluoride suppresses spleen development through MAPK/ERK signaling pathway in mice

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ABSTRACT

Numerous studies have documented that excessive fluoride intake could cause pathological damage and functional disorder in organisms. Nevertheless, the systemic mechanism of fluorosis inhibiting the proliferation and development of splenic cell is still scarce. The preliminary studies have confirmed that high-dose NaF could inhibit splenic lymphocytes proliferation in vitro and cause toxic effects on spleen development in vivo. Here this study continued to explore the signaling pathway with the methods of quantitative real-time polymerase chain reaction (qRT-PCR) and western blot (WB), revealing the mechanism of fluorosis in the growth system. Mice in 4 groups (control, 12 mg/kg, 24 mg/kg, 48 mg/kg) were gavage administrated with NaF solution continuously for 42 days. The results suggested that NaF more than 12 mg/kg slowed down the growth of mice, inhibited spleen growth and development, which was characterized by decreasing spleen volume, and inducing splenic cell apoptosis. For the Ras-Raf-MEK-ERK signaling pathway, the mRNA and protein expression levels of Ras were significantly elevated, and the phosphorylated protein expression levels of Raf (B-Raf, C-Raf) were increased. Meanwhile, mice mRNA expression levels were increased in a time and dose-dependent manner on the 21st and 42nd days of the experiment. Additionally, the mRNA and protein levels of MEK1/2 were increased on the 21st day of the experiment, while reduced on the 42nd day. The ERK1/2 levels were significantly decreased at both 21st and 42nd days of the experiment. This study showed that NaF activated Ras to induce downstream Raf-MEK-ERK cascade reaction, but failed to activate ERK eventually, the proliferation signal from the cell surface could not transmit to the nucleus, interfering with the regulation of cell proliferation, differentiation, meiosis, and suppressed spleen development ultimately.

1. Introduction

Fluoride distributes in the natural environment extensively and is used in industry, agriculture as well as medicine widely (Jha et al., 2011; Strunecká et al., 2004). Also, fluorine is an essential trace element for human health to maintain the teeth, bones and medical chemistry uses (Ericsson, 1970; Gillis et al., 2015; Hagmann, 2008). The concentration of fluoride in water at environment is between 0.1 and 1.5 ppm, which is given by world health organization (WHO) guidelines(Tomoki Nishimura et al., 2013). Excessive fluoride intake induces pathological lesions and dysfunction of many tissues and cells (Basha and Sujitha, 2012; Ersoy et al., 2010; Wang et al., 2002; Zhao et al., 2014). Numerous studies have shown that fluoride causes cytotoxicity, apoptosis and DNA damage in human beings and animals (Matsui et al., 2007; Nguyen Ngoc et al., 2012; Song et al., 2014, 2002; Wang et al., 2004). Previous studies have also proved that a higher fluoride intake could inhibit the development of the spleen by inducing cell apoptosis and autophagy (Deng et al., 2016a, 2016b, 2017a; Kuang et al., 2018; Tao et al., 2009), blocking cell cycle progression and interdicting cell proliferation in vitro (Chen et al., 2009; Kuang et al., 2016a, 2016b). Comprehensive analysis of the existing reports, the systematic mechanism of fluorosis inhibiting the development of the spleen is still scarce. To explore the molecular mechanism of fluoride in the growth system, further research on the signal transduction pathway is needed.

Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases; their signal transduction exists in most cells;

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they are activated by an enormous array of stimuli and are involved in a wide range of processes ranging from proliferation, differentiation to apoptosis (Qi and Elion, 2005a; Sun et al., 2013). There are five families of MAPKs in mammalian cells: extracellular signal-regulated kinases (ERK1 and ERK2); Jun N-terminal kinases (JNK1, JNK2 and JNK3); p38 kinase isozymes (p38α, p38β, p38γ, and p38δ); ERK3/ERK4; and ERK5 (Chang and Karin, 2001). Among these subfamilies, the MAPK/ERK pathway (also known as the Ras-Raf-MEK-ERK pathway) could transmit signals from cell surface receptors into the nucleus, thereby regulating cell proliferation, cell differentiation and meiosis (Qi and Elion, 2005b). Briefly, the mitogens and growth factors activate receptor tyrosine kinase, which dimerizes and activates its downstream small GTP binding protein (GTPase) Ras (Meloche and Pouysségur, 2007). And then trigger a series of phosphorylation events of MAPK cascade (RAF-MEK-ERK), induce ERK phosphorylation and activation ultimately. Activated ERK further induces downstream phosphorylate targets, including two critical targets: Cyclin D-CDK4 and Cyclin D-CDK6 complexes, which are involved in cell proliferation regulation(Chambard et al., 2007). The cell cycle transition from G1 to S phase is regulated by Cyclin D-CDK4/6 and Cyclin E/A-CDK2 complexes, when cells are ready to enter the S phase in response to mitogen, they will increase in the late G1 phase(Sánchez and Dynlacht, 2005). It is necessary to activate ERK1/2 of Ras downstream signal by mitogen, which could eliminate cell cycle arrest and allow cell proliferation into the S phase.

MAPK/ERK pathway is related to lymphocyte development and cytokine production, and participates in the regulation of cell growth and differentiation(Dong et al., 2002; Yue et al., 2015). Natalia et al. (2006) revealed the mechanism of NaF-induced MAPK activation in pulmonary artery endothelial cells: Ras effectively combined with fluoride to activate Raf-1 quickly, triggering the Ras/Raf-1/MEK/ERK cascade (Bogatcheva et al., 2006). Chen et al. (2014) pointed out that fluoride inhibits the expression of p-ERK protein through the ERK pathway, resulting in kidney injury in carp (Chen et al., 2014). Zhao et al. reported that NaF treatment resulted in the inhibition of phosphorylation of MEK and ERK1/2 (p-MEK and p-ERK1/2) in mouse ameloblasts (LS8 cells), suggesting that high-dose fluoride may affect ERK signal transduction, thus affecting the differentiation of ameloblasts (Zhao et al., 2021).

Additionally, numerous researchers also reported that sodium fluoride (NaF) influenced organ development, cell cycle progression, promoted cell apoptosis, caused damage and dysfunction via ERK signaling pathway (Chen et al., 2014; Geng et al., 2014; Ma et al., 2017; Zhu et al., 2017). Based on previous research, this study further to explored how NaF influenced spleen development, to clarify and confirm the underlying mechanism of MAPK/ERK in spleen development. Results showed that NaF treatment influenced spleen development, which is characterized of spleen volume, growth index, cell cycle (associated key regulatory molecules) and combined with previous research results in vitro/vivo (Kuang et al., 2016b, 2017), revealed that NaF treatment suppresses spleen development through MAPK/ERK signaling pathway in mice.

2. Results

2.1. Clinical symptoms

The mice's appetite dramatically declined at 24 mg/kg and 48 mg/kg groups start over day 14. From day 35, mice in the 12 mg/kg group had normal performance, 24 mg/kg and 48 mg/kg groups had a fecal stench, 48 mg/kg group had shortness of breath, circular movement, extreme sensitivity to noise and restlessness.

Compared to the control group, the weight of mice increased slowly in three (3) NaF-treatment groups (Fig. 1A). Simultaneously, spleen volume in three (3) experimental groups decreased (Fig. 1B-C) and the spleen organ index was significantly lower in the 24 mg/kg and 48 mg/kg groups than the control group on the 21st and 42nd day of the experiment(Ping Kuang, 2017).

2.2. Ultrastructural changes in the spleen

On the 42nd day of the experiment, compared with the control group, apoptotic cells were found in three NaF-treatment groups with a

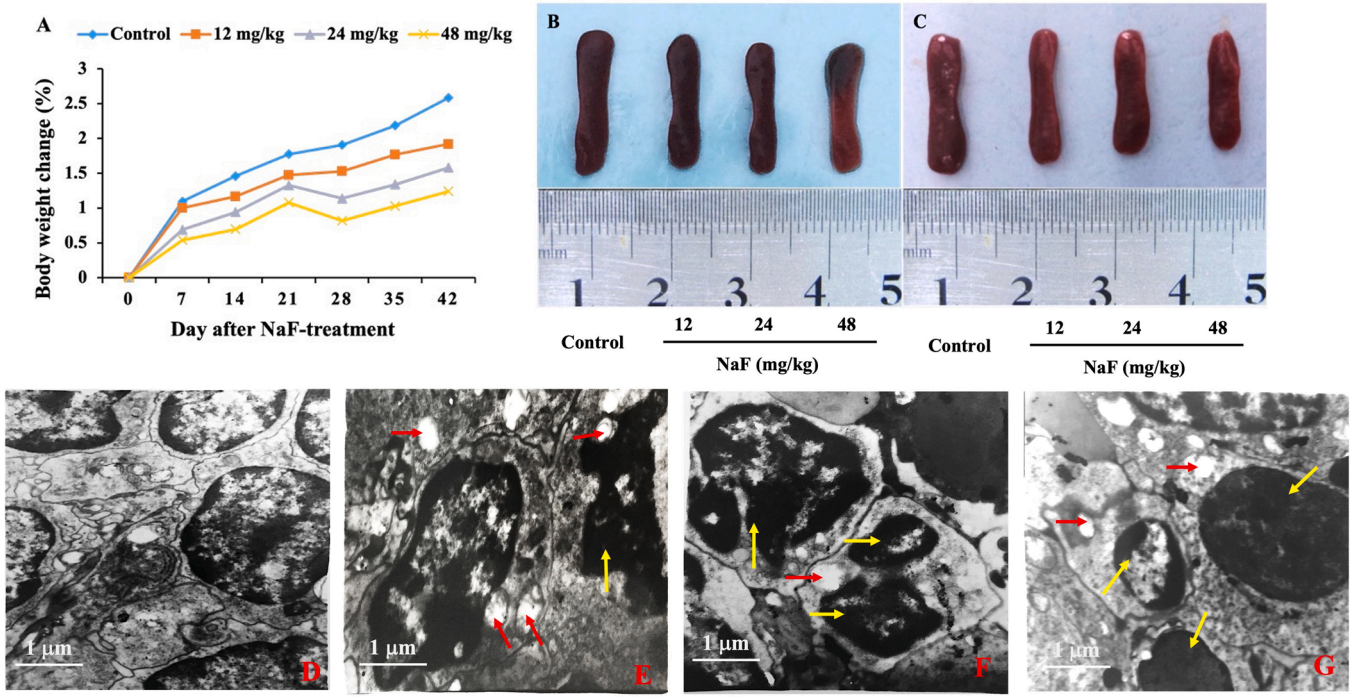

Fig. 1. The clinical symptoms and ultrastructural changes. A: body weight growth rate (%) in mice after NaF-treatment; B-C: change of spleen size on day 21st (B) and day 42nd (C) (N = 8); D-G: splenic ultrastructural change on day 42nd, Yellow arrow: apoptotic cells ; Red arrow: mitochondria; From left to right: Control group, 12 mg/kg group, 24 mg/kg group, 48 mg/kg group.

dose-dependent increasing trend (the yellow arrow indicates the change of apoptotic cells, and the red arrow indicates the change of mitochondria). Results are shown in Fig. 1D–G.

Changes in apoptotic cells: chromatin condensation was circular, C-shaped, semicircular, or crescent attached to the nuclear membrane, and the electron density in the center of the nucleus was low; the nuclear chromatin was completely condensed to form round, and oval or cord-like dense spots with high electron density.

Changes in mitochondrial: swelling in varying degrees, cristae fracture or even dissolution in vacuole shape.

2.3. Changes of cell cycle-related regulatory molecules in spleen

Previous study confirmed that NaF inhibited splenic cell cycle progress in mice, which was characteristic by increasing the cell percentages of G0/G1 phase, and decreasing proportion in S and G2/M phases when compared to the control group (Ping Kuang, 2017). Additionally, the cyclin D-dependent kinases (CDK4/6 or CDK2) bound to cyclin D or cyclin E/A could regulate cell cycle progression from G1 to S phase (Sánchez and Dynlacht, 2005). Previous results showed that the CDK2 and CDK4 protein levels were significantly decreased except for CDK1, the protein levels of cyclin D and cyclin E were also significantly reduced except cyclin A and cyclin B when compared to the control group(Ping Kuang, 2017). Here, this study continued to detect their mRNA expression levels.

On the 21st and 42nd days of the experiment, the mRNA expression levels of cyclin-dependent kinase CDK2 and CDK4 in 12 mg/kg, 24 mg/kg and 48 mg/kg groups decreased significantly ($P < 0.01$ or $P < 0.05$), but there was no significant change in CDK1 mRNA level (Fig. 2A–C).

The mRNA expression levels of cyclin D1 and cyclin E1 in three (3) NaF-treatment groups were significantly lower than those in the control group ($P < 0.05$ or $P < 0.01$), but there was no significant difference in cyclin A2 and cyclin B1 mRNA levels (Fig. 2D-G).

2.4. Changes of MAPK/ERK signaling pathway regulatory molecules in the spleen

Compared to the control group, on day 21 and day 42, the mRNA expression level of k-Ras was significantly increased ($P < 0.05$ or $P < 0.01$); whilst its protein expression levels increased in three NaF treated groups at 21st day of the experiment, in 24 mg/kg and 48 mg/kg groups elevated at 42nd day of the experiment ($P < 0.05$ or $P < 0.01$). Results are shown in Fig. 3A–C.

On the 21st and 42nd days of the experiment, the mRNA expression levels of B-Raf in 24 mg/kg and 48 mg/kg groups were significantly elevated ($P < 0.05$ or $P < 0.01$) when compared to the control group, and its protein expression levels of phosphorylated B-Raf (Ser445) were dramatically increased ($P < 0.05$ or $P < 0.01$) in the 24 and 48 mg/kg groups at 21st day, and in the 12, 24 and 48 mg/kg groups at 42nd day of the experiment when compared to the control group (Fig. 3A-B, D).

Also observed higher mRNA expression levels of C-Raf ($P < 0.05$ or $P < 0.01$) in the 48 mg/kg group on day 21, and in 12, 24 and 48 mg/kg groups on day 42 when compared to the control group (Fig. 3E: upper and middle panel). Additionally, phosphorylated protein expression levels of C-Raf (Ser289/296/301) were significantly increased ($P < 0.05$ or $P < 0.01$) in 24 and 48 mg/kg groups on day 21, and 12, 24 and 48 mg/kg groups at day 42 (Fig. 3E: lower panel).

A significant increase in MEK1/MEK2 mRNA expression level was noted in the 48 mg/kg group on the 21st day of the experiment ($P < 0.01$). Conversely, a marked decrease ($P < 0.05$ or $P < 0.01$) in the 24 and 48 mg/kg groups on the 42nd day of the experiment (Fig. 4C: upper and middle panel). Similarly, the phosphorylated MEK1/2 (Ser217/221) protein expression tendency was consistent with the mRNA, which was significantly increased ($P < 0.01$) in 24 and 48 mg/kg groups on the 21st day of the experiment and significantly decreased ($P < 0.01$) in the 12, 24 and 48 mg/kg groups at 42nd day of the experiment when compared to the control group (Fig. 4A–B, C: lower panel).

From Fig. 4D: upper and middle panel, the mRNA expression levels of ERK1/ERK2 were significantly decreased ($p < 0.01$) in 12, 24 and 48 mg/kg groups on both the 21st and 42nd days of the experiment. Meanwhile, the phosphorylated protein expression levels of ERK1/2 (Thr202/Tyr204) were significantly decreased ($P < 0.05$ or $P < 0.01$) in the 24 and 48 mg/kg groups on the 21st day and in three NaF-treated groups on the 42nd day of the experiment when compared to the control group (Fig. 4A–B, D: lower panel).

3. Discussion

The preliminary works had been documented that NaF causes toxic effects on spleen development in mice, including splenic lymphocytes cell cycle arrest, cell proliferation inhibition, cell apoptosis and

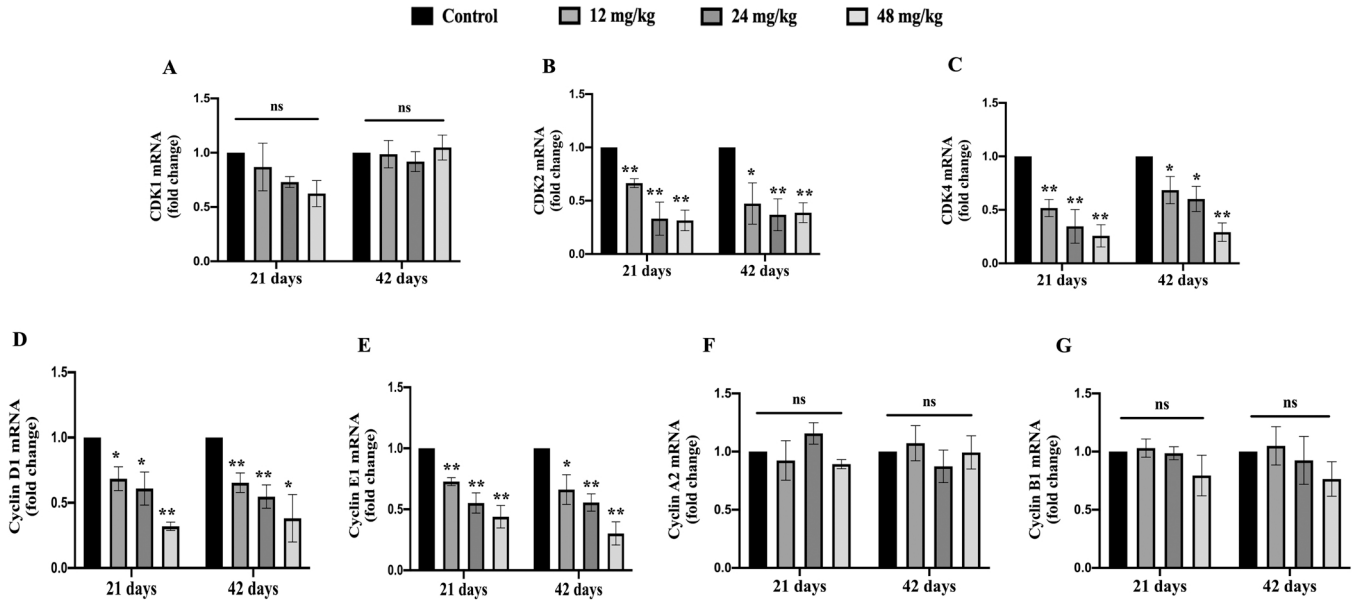

Fig. 2. Changes of splenic CDKs(A-C) and Cyclins(D-G) mRNA expression levels at 21st and 42nd day of the experiment.

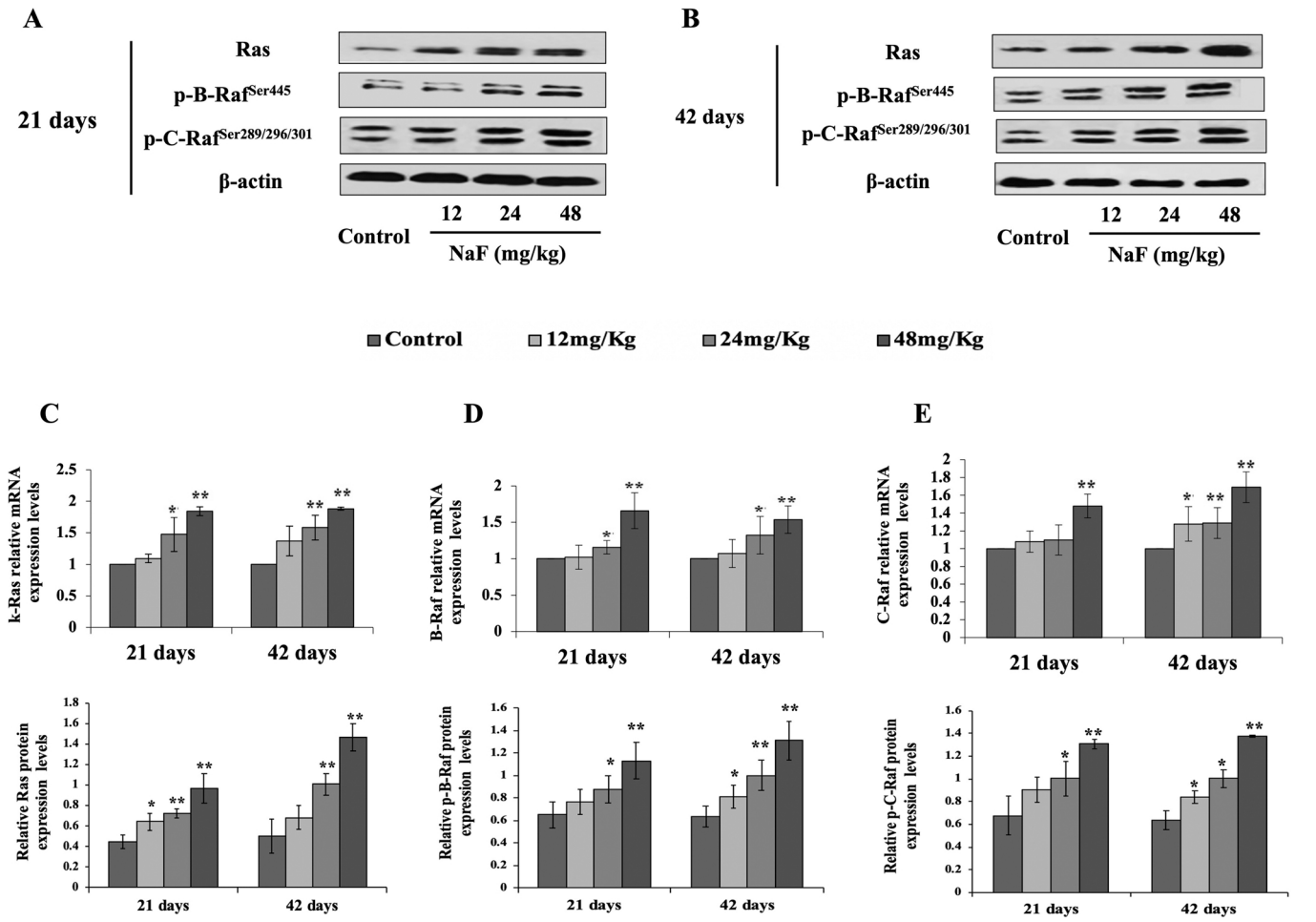

Fig. 3. Changes of splenic mRNA and protein expression levels of Ras, B-Raf and C-Raf at 21st and 42nd day of the experiment. A-B: The western blot assay; C-E: The mRNA and protein expression levels of Ras, B-Raf and C-Raf. upper panel: quantitative analysis of mRNA expression; lower panel: quantitative analysis of protein expression.

autophagy (Deng et al., 2017b; Kuang et al., 2016b, 2017, 2018). In addition to previous results, this study further confirmed that mice grew slowly, the spleen volume and organ index decreased, ultrastructural observation of the spleen showed that the number of apoptotic cells increased in a dose-dependent manner in NaF treatment groups, the mitochondria swelled, cristae broke or even disappeared and showed vacuolation, which indicated that growth and development of spleen were inhibited (Fig. 1). Also, it has been reported that fluoride treatment could cause spleen pathological injury and dysfunction in rats (Das et al., 2006).

The cell cycle is involved in the regulation of cell proliferation, differentiation, body growth and development, when it is inhibited, the ability of cell proliferation is weakened, and the development of tissues/organs is blocked (Dalton, 2015). We had documented splenic lymphocytes cell cycle was inhibited at G0/G1 phase by NaF treatment in vivo and in vitro (Kuang et al., 2016b, 2017). The cell cycle progress is regulated by specific types of cyclins and cyclin-dependent kinases (CDKs) (Sánchez and Dynlacht, 2005). Progression from G1 to S phase of the mammalian cell cycle is regulated by Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes (Baldin et al., 1993), S phase is mainly regulated by Cyclin A-CDK2, and the G2/M phase is primarily regulated by Cyclin B-CDK1, the decrease of their activity could induce cell cycle arrest (Wang et al., 2014). Our research showed that NaF over 12 mg/kg, the mRNA and protein(Ping Kuang, 2017) expression levels of Cyclin D, CDK4, Cyclin E and CDK2 were decreased significantly. Still, there was no significant difference in Cyclin B, CDK1 and Cyclin A, which confirmed that G1 checkpoint activity of cell cycle was inhibited, so resulted in cell cycle blocked in G1 phase by NaF treatment (Fig. 2).

It is well known that Mapk/Erk pathway (also known as Ras-Raf-Mek-Erk) is related to cell proliferation, differentiation, migration, aging and apoptosis (Sun et al., 2015), and also has been clarified that this pathway plays an essential role in lymphocytes development (Dong et al., 2002). Recent studies have shown that cell cycle transition from G1 to S phase requires continuous activation of ERK (Jones and Kazlauskas, 2001; Yamamoto et al., 2006). Thus, to explore whether Mapk/Erk signaling pathway is involved in splenic cell cycle arrest by NaF, we detected the mRNA and protein expression levels of Ras and Raf firstly, the results showed that NaF more than 12 mg/kg, the protein expression levels of k-Ras、p-b-raf、p-c-raf and mRNA expression levels of Ras、b-raf、c-raf were significantly elevated. Hirakawa et al. (1988) reported that unless there is a functional cooperative oncogene, the expression of activated Ras could induce cell growth arrest (Hirakawa and Ruley, 1988). The activation of Ras could cause downstream Raf activates and blocks the cell cycle progression, which may be consistent with that discussed by Sewing and Woods et al., that is high-intensity Raf signal transduction causes cell cycle arrest by inducing the prominent expression of cell cycle inhibitor (cdki) $p21^{cip1}$ (Sewing et al., 1997; Woods et al., 1997). Meanwhile, CDK inhibitor $p21^{cip1}$ could also mediate the continuous activation of MEK/mitogen activated protein kinase pathway, resulting in the inhibition of CDK activity and growth arrest (Pumiglia and Decker, 1997). ERK is activated by phosphorylation on threonine/tyrosine residues by MEK, the activated ERK transfers from cytoplasm to nucleus and activates downstream nuclear targets, including G1 phase cyclins (cyclin D and cyclin E) (Torii et al., 2006).

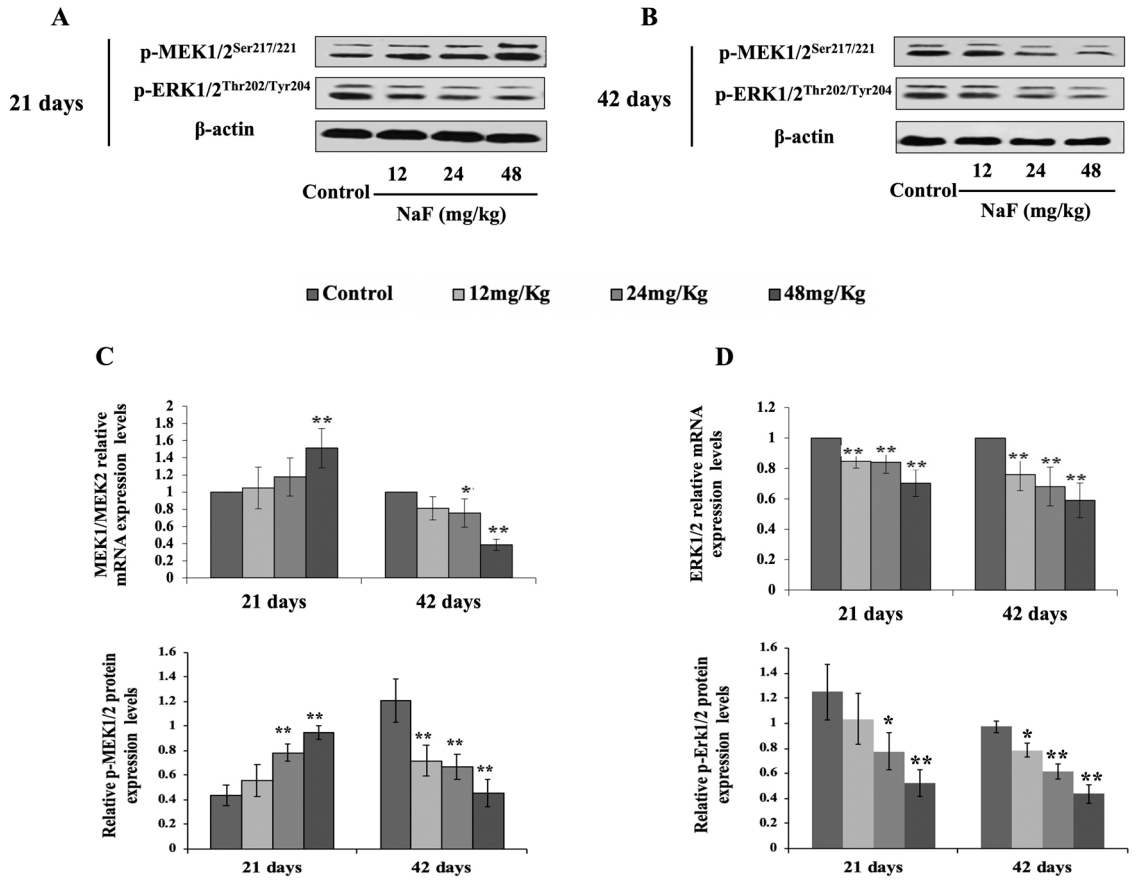

Fig. 4. Changes of splenic mRNA and protein expression levels of MEK1/2 and ERK1/2 at 21st and 42nd day of the experiment. A-B: The western blot assay; C-D: The mRNA and protein expression levels of MEK1/2 and ERK1/2. upper panel: quantitative analysis of mRNA expression; lower panel: quantitative analysis of protein expression.

Here, mRNA level of MEK1/MEK2 and protein level of p-MEK1/2 was increased after NaF treated on the 21st day of the experiment, with experiment progressing to day 42, its mRNA and protein expression levels showed significant decrease trends, at the same time, the mRNA level of ERK1/ERK2 and protein level of p-ERK1/2 decreased significantly. These results indicated that NaF treatment activated Ras on the surface of spleen lymphocytes and induced a series of phosphorylation cascade reactions of downstream (Raf-Mek-Erk) signals but failed to phosphorylate and activate ERK ultimately.

ERK activation is related to the induction of cyclin D expression. Lavoie et al. (1996) confirmed that blocking ERK activity by expressing dominant negative MEK can reduce cyclin D1 (Lavoie et al., 1996), and only sustained activation of ERK can induce cyclin D1 continued expression in G1 phase (Weber et al., 1997). In addition, the regulation of CDK2 by cyclin E contributes to the G1/S transition, and the formation of cyclin E-CDK2 complex is also indirectly regulated by ERK (Sherr and Roberts, 1999). Whether CDK2 can be transcribed correctly in the nucleus depends on ERK activity, it can regulate the phosphorylation of CDK2 threonine site 160 (CDK2 activation site), the nuclear localization of CDK2 could be changed if ERK activation was blocked (Keenan et al., 2001). In other words, the inactivation of ERK inhibits the formation of cyclin D1-CDK4 complex and causes the nuclear translocation of CDK2. Finally, the cell proliferation signal cannot enter the nucleus correctly, induced cell cycle blocked in G1 phase (Figs. 3–4).

4. Conclusion

NaF activated Ras on the surface of splenic cells to induce downstream RAF-MEK-ERK cascade reaction but failed to phosphorylate and activate ERK eventually in mice. Which resulted in the proliferation signal could not transmit from cell surface to nucleus, thus, to further interfere or affect the process of normal cell meiosis, cell proliferation and differentiation (inhibition of the CDK4-cyclin D and CDK2-cyclin E complexes activity, blocking the process from G1 to S phase). Finally, splenic cell proliferation obstruction and cell cycle arrest in G1 phase (Fig. 5) .

5. Materials and methods

5.1. Animals and treatment

A total of 240 ICR mice (3-week-old, male/female, 18–20 g) were from DOSSY Experimental Animal Corporation in Chengdu, China, and were divided into four groups ($N = 60$) randomly. Food and water were provided ad libitum. Mice were gavage administrated with NaF solution (12 mg/kg, 24 mg/kg, 48 mg/kg) in experimental groups and distilled water in control group at the same time. The intragastric dose was 1 mL/100 g body weight once daily for 42 days.

All animal and experimental procedures were approved by the Animal Care and Use Committee at Sichuan Agricultural University under standard protocol.

5.2. Clinical observation

The clinical manifestations of the growth and development of mice were observed and recorded every day during the experiment. The mice were weighed on the days 0, 7, 14, 21, 28, 35 and 42 of the experiment, and their body weight changes were recorded and counted.

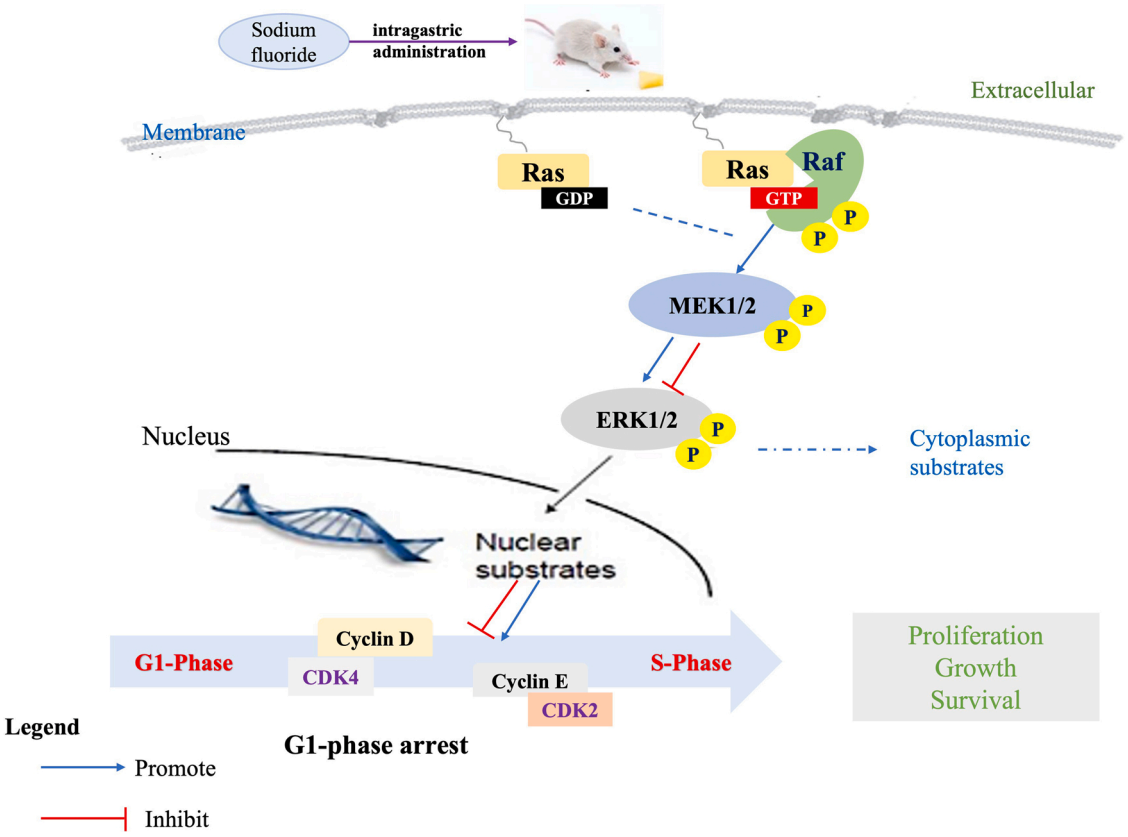

Fig. 5. Schematic diagram of NaF-induced G1 phase arrest of spleen in mice.

5.3. Ultrastructural observation of spleen

On the 42nd day of the experiment, four (4) mice were selected from each group for dissection, removed the spleen quickly and cut into 1 mm x 1 mm x 1 mm tissue blocks, then fixed in 2.5 % glutaraldehyde (pH 7.2). After fixation, rinse twice with 0.2 M phosphate buffer with pH 7.2

Table 1
Sequence of primers used in qRT-PCR.

Gene symbol	Accession number	Primer	Primer sequence (5'−3')	Product size	Tm (°C)
CDK1	NM-007659.3	Forward	AAGTGTGGCCAGAAGTCGAG	97 bp	60
		Reverse	TCGTCCAGGTTCTTGACGTG		
CDK2	NM-016756	Forward	TTGGAGTCCCTGTCCGAACT	142 bp	60
		Reverse	CGGGTCACCATTTCAGCAAAG		
CDK4	NM-009870	Forward	CAATGTTGTACGGCTGATGG	120 bp	60
		Reverse	GGAGGTGCTTTGTCCAGGTA		
Cyclin D1	NM-007631.2	Forward	CAAGTGTGACCCGGACTGC	169 bp	61
		Reverse	CACATCTCGCACGTCGGT		
Cyclin E1	NM-007633.2	Forward	CTTATGGTGTCCTCGCTGCT	143 bp	59
		Reverse	TCCCATCTCCCGGATAACCA		
Cyclin B1	NM-172301.3	Forward	TGCGCCTGCAGAAGAGTATC	135 bp	60
		Reverse	CCAGTCACTTCACGACCCTG		
Cyclin A2	NM-009828.2	Forward	GTCAACCCCGAAAAACTGGC	122 bp	60
		Reverse	TTAAGAGGAGCAACCCGTCG		
K-Ras	NM-021284.6	Forward	GATGTGCCTATGGTCCTGGT	144 bp	62
		Reverse	GGCATCGTCAACACCCTGTC		
B-Raf	NM-139294.5	Forward	CCACAGATGCATCACGGAAC	97 bp	62
		Reverse	CATCTTGCGGGTACCACTGT		
C-Raf	NM-029780.3	Forward	ACTCCAGAGCAACTTCAGGC	85 bp	60
		Reverse	ACCCCATGAACAGCAGGATG		
MEK1	NM-008927.3	Forward	TGCCCAAGAAGAAGCCGAC	131 bp	60
		Reverse	CTCGTCAAGCTCCAGCTCC		
MEK2	NM-001347144.1	Forward	CCACCTGATGCCAAGGAACT	129 bp	59
		Reverse	GTCCATCCCATGACCACTGA		
ERK1	NM-011952.2	Forward	CACTGGCTTTCTGACGGAGT	136 bp	60
		Reverse	CCGGTTGGAGAGCATCTCAG		
ERK2	NM-001038663.1	Forward	TCCAACCTCCTGCTGAACAC	120 bp	62
		Reverse	CCAACGTGTGGCTACGTACT		
β-actin	NM-007393	Forward	GCTGTGCTATGTTGCTCTAG	117 bp	60
		Reverse	CGCTCGTTGCCAATAGTG		

(15 min/time); fixed with 1 % osmium tetroxide for 1 h; dehydrate step by step in gradient ethanol, and then embed (the embedding agent is pure acetone: epoxy resin); place the sample in a constant temperature incubator (35 °C for 24 h and 60 °C for 17 h); at last, prepare ultrathin sections, install them on the copper mesh, dye the samples with uranyl acetate and lead acetate at 25 °C for 15–20 min, to transmission electron microscope observation, recording and photographing.

5.4. Determination of mRNA expression levels of cell cycle regulatory molecules and MAPK/ERK signaling pathway molecules in the spleen by qRT-PCR

On the 21st and 42nd days of the experiment, extracted spleens from eight mice/group and homogenized them by mortar and pestle after stored in liquid nitrogen. Extracted total RNA (9108/9109, Takara, Japan), synthesized cDNA with Prim-Script™ RT reagent Kit (Takara, Japan) and used it as a template for qRT-PCR ultimately. The specific gene primers were designed by Primer 5 software and synthesized in Sangon (Shanghai, China) (Table 1).

All qRT-PCR reactions were performed by LightCycler 96 (Roche, Germany) using SYBR® Premix Ex Taq™ II (Takara, Japan). Each melting curve ensured a single peak for each PCR product and is verified by agarose gel electrophoresis. Here, the internal reference was mouse β-actin and the genes' relative expression was analyzed by the $2^{-\Delta\Delta CT}$ method(Livak and Schmittgen, 2001).

5.5. Determination of protein expression levels of MAPK/ERK signaling pathway molecule in the spleen by Western blot

On the 21st and 42nd days of the experiment, spleen samples of eight mice/group were taken to detect the protein expression levels of MAPK/ERK signaling pathway by western blot.

Extracted protein with RIPA lysis buffer (Beyotime, China), then quantity it with BCA Protein Assay Kit (Beyotime, China). Loading protein 30 μg to SDS-PAGE and transferring to nitrocellulose filter membranes. Blocking membrane for 1 h with 5 % fat-free milk and incubating with primary antibodies at 4 °C overnight. The primary antibodies were Ras, MEK1/2, ERK1/2, B-Raf, C-Raf (CST, USA), incubating with secondary antibody for another 1 h and washing membrane with PBS-tween till visualizing by ECL™ (Bio-Rad, Hercules, CA, USA). The protein blots were quantified by Image J software.

5.6. Statistical analysis

The data were statistically analyzed by Graphpad prism 8 software, each group was calculated by mean ± standard deviation. Differences between groups were compared by one-way ANOVA. $P < 0.05$ or $P < 0.01$indicates significant differences.

CRediT authorship contribution statement

Ping Kuang, Hengmin Cui and Li Yu designed the experiments, Ping Kuang carried out the experiment, analyzed and interpreted the data. Ping Kuang, Hengmin Cui and Li Yu wrote and revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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